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FINAL REPORT

"RESEARCH ON THE ADRENAL CORTICAL HORMONES AND HYALURONIDASE"

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FINAL REPORT

Condensed digest of related past and present work done under the sponsorship of the Office of Naval Research, relative to the pituitary, placenta, and adrenal-hyaluronidase relationships.

We have investigated the relationships between the various hormones of the adrenal cortex, the enzyme hyaluronidase, and its substrate hyaluronic acid, with specific reference to tissue permeability (9-14, 16). In addition, during the past four years, this work has been carried out with a view towards its possible relation to the field of rheumatoid and other collagen disease processes (15, 17, and data in process of compilation for publication). As the studies progressed, several problems presented themselves: a. Which fractions of the adrenal cortex were responsible for the results obtained. b. What was the mechanism or mechanisms of action involved. c. In what way was the hyaluronic acid metabolism involved in the arthritides and in other collagen diseases. A review of certain of these studies was presented in part, in the book "The Adrenal Cortex", 2nd transactions, J. Macy Foundation, 1951 (16). The practical implications of these findings in the field of collagen diseases are obvious, and further investigations in many phases of these studies will be discussed in other parts of this report.

II

Our experimental observations indicated a specific relationship between hyaluronidase inhibition and chemical structure of the adrenal cortex steroids. In a series of *in vivo* studies, several pure steroids were investigated in an attempt to define more accurately the structural or hormonal factors that are related to the inhibition of the hyaluronidase-enhanced spreading phenomenon (12). The experimental evidence indicated that inhibition of the spreading reaction by the adrenal hormones is restricted to steroids that have an oxygen at Carbon 11, notably Compounds E, F, and to a lesser extent, Compound A. DOCA, the amorphous fraction, related sex steroids and growth hormone, have consistently been found to be without effect. *In vitro* studies have appeared to bear out this conclusion,

and experimental evidence suggested a direct action of these adrenal hormones on the enzyme hyaluronidase in vitro (16, and unpublished studies).

III

Related studies on the role of environmental temperature and humidity in these processes were in part reported, as suggestive that increased environmental temperature and humidity play a role in stimulating the adrenals and thus causing a decrease in the permeability of the ground substance, (10).

IV

Investigations on the adrenal-hyaluronidase relationship in infection, pointed out that with hyaluronidase-containing bacteria, the course of infection could be inhibited markedly in normal animals as well as in adrenalectomized animals by the administration of adrenal cortical extracts; whereas in the case of the hyaluronidase-free bacteria and viruses, a protective effect of adrenal cortical extract could be demonstrated only in the adrenalectomized animals. With both types of organisms, replacement therapy in the adrenalectomized animals did not give as complete protection against infection as was found in the normal control animals. It was concluded that while the adrenal-hyaluronidase relationship is one of the factors involved in the lowered resistance of animals to infection, a lack of hyaluronidase inhibition can be only one component of this complicated deficiency condition (14, and unpublished studies).

V

We have shown that either adrenocorticotrophic hormone or the C-11 oxygenated steroids inhibited the hyaluronidase-enhanced spreading phenomenon in the rabbit and in the mouse (9-16). In view of the favorable influence of pregnancy on certain of the collagen diseases, it was of interest to determine whether chorionic gonadotrophins had any influence on this phenomenon. This was found

to be the case (CG), but in the course of these investigations it became apparent that the inhibition produced by this agent was due, not to the gonad-stimulating principle, but to a contaminant which exhibited many of the properties previously associated with ACTH from pituitary tissue (15). Several similarities in the biologic activity of ACTH, CG, and CG which had been heat-inactivated to destroy its gonadotrophic activity (HICG) from human pregnancy urine have been shown. Furthermore, it was demonstrated that CG exhibits ACTH-like activity which could not be associated with its gonadotrophic activity. The following lines of evidence established this similarity: Both chorionic gonadotrophin and heat-inactivated chorionic gonadotrophin caused a marked inhibition of hyaluronidase-enhanced spreading in normal and castrate animals of both sexes in the presence of the adrenals but were without effect in the adrenalectomized animals. This effect was qualitatively identical to that found with pituitary ACTH. The possibility that this effect was due to non-specific toxicity or mediation through the gonads was ruled out by numerous control studies. In the hypophysectomized animals, both CG, HICG, and ACTH were found to exhibit the same marked degree of inhibition of hyaluronidase activity. There were two possibilities as to the source of the ACTH-like activity found in these preparations from human pregnancy urine. The first, that the process of extraction included such quantities of pituitary ACTH as may have been excreted in the urine; the second, that the placenta may normally have formed the active agent, a portion of which was also excreted in the urine. These investigations opened new lines of investigations, and suggested the possibility that ACTH of human origin might be obtained for clinical use, thus providing another source to supplement the quantities now available only from animal sources.

VI

Preliminary investigations of placental extracts proved informative from several standpoints and although the crude methods of isolation that were employed

did not permit quantitative interpretations of yields or of purity, results of these studies indicated that significant quantities of ACTH-like material are produced by the placenta (17). Furthermore, evidence was cited indicating the lack of measurable quantities of the C-11 oxygenated steroids in these tissue extracts.

In some of our studies, a modification of Lyon's procedure for the extraction of mammotropic hormone from pituitary tissue was employed; however, because of the high water content of the placental tissue and blood and the likelihood that the major portion of the ACTH activity might remain in the first acetone residue normally discarded in obtaining the active fraction (Fraction I), further extraction of the residue was made and labelled Fraction II. Since Fraction II, represents material that is normally discarded in the Lyon's procedure for extraction of pituitary tissue, it was most interesting that Fraction II of the placenta consistently showed a greater degree of activity than did Fraction I, and that more than twice as large a yield of active dry powder was hereby obtained. When fresh placental tissue was extracted, the fractions were not only extremely large for the crude procedures employed in extraction, but also possessed ACTH activity in terms of hyaluronidase inhibition, in dosage ranges comparable to those for pituitary preparations. However, placental tissue that had been frozen and stored prior to extraction did not yield fractions comparable in activity or quantity to those obtained with the use of fresh placenta. Thus, a destruction of ACTH activity was indicated, even though the tissue remained in the frozen state. (Astwood has observed this liability of ACTH in the isolation of pituitary tissue.)

Identification of the type of activity found in the various fractions of placental extracts were based largely upon previous work from this laboratory which has shown the adrenal steroid-hyaluronidase relationship to be a normally functioning physiological mechanism. Further, that certain of the pharmacologic

effects observed following administration of large quantities of these hormones, are related to their influence on this complex enzyme system. In these investigations ACTH was shown to be a potent inhibitor of the hyaluronidase-enhanced spreading phenomenon presumably through the release of effective C-11 adrenal steroids; and evidence of a correlation of the C-11 adrenal steroids in inhibiting the hyaluronidase-enhanced spreading phenomenon, and inhibition of the release of reducing sugars from hyaluronic acid by inactivation of hyaluronidase in vitro has been obtained.

The markedly similar hyaluronidase-inhibitory effects produced by the various placental extract fractions, by the human chorionic gonadotrophins, and by the human heat-inactivated chorionic gonadotrophin preparations, compared favorably with the effects produced by the standardized commercial ACTH preparations from the pituitary in normal, adrenalectomized, and hypophysectomized mice. The placental tissue fractions caused a marked inhibition of hyaluronidase in both normal and hypophysectomized animals, and with the larger dosages of crude powders, this inhibition approximated 100% of added enzyme. In all cases, the placental extracts were inactive in adrenalectomized animals.

The possibility that the observed activity was due in any way to the presence of steroids of the nature of cortisone was ruled out by the fact that enzyme inhibition was not observed in adrenalectomized or hypophysectomized-adrenalectomized animals, and by the fact that the placental tissue extracts were without effect when administered intradermally at the site of hyaluronidase injection.

The factors of non-specificity were ruled out by the lack of effectiveness of tissue extracts and serum albumin, and further supporting this conclusion were the results of numerous studies carried out in this laboratory that have shown that toxic substances such as formaldehyde, spermine, colchicine and bacterial, as well as other agents, do not elicit an inhibition of hyaluronidase in the

normal or adrenalectomized animals. Although not directly related to the establishment of ACTH activity in the placental extracts, it is of importance in studying mechanism of action, that epinephrine in amounts of 0.02 mg. or less per mouse did not influence the spreading reaction, and only when doses of 0.05 mg. were employed was any degree of inhibition noted and then only in animals with intact adrenals (unpublished data).

These studies on ACTH-like activity of placental tissue indicated that the placenta contains relatively large amounts of the hormone. In addition, even though the preparations were extremely crude, there was a striking lack of toxicity with their administration. For example, very large doses of placental extracts could be administered to adrenalectomized or hypophysectomized mice without eliciting signs of acute toxicity, while commercial pituitary ACTH preparations in the same or smaller dosages proved toxic under these conditions. Since the material is of human origin and presumably would not cause a foreign protein reaction when administered to humans, it is possible that placental ACTH would have an important practical value as there have been an increasing number of reports of sensitivity to pituitary ACTH preparations. The observation that ACTH activity is obtained with placental extracts is in agreement with the previous studies from this laboratory that human pregnancy urine preparations heated to destroy gonadotrophic activity, possess ACTH activity (15), and it is probable that the placental extracts contained the precursor of these excretory products. (Note: Venning and others have established evidence that urinary corticoids increase during pregnancy; Dobriner and others have shown there is a marked increase in urine content of a variety of steroids following ACTH administration; it would be of interest and of importance to establish whether a similar urinary steroid pattern would result following administration of placental extracts in non-pregnant conditions.)

Inasmuch as the summarized experiments definitely showed that there was an

adrenal trophic effect, and since epinephrine was excluded from consideration as a mediator of this action, it was concluded that the effect in all probability was trophic for the adrenal cortex. It was therefore quite appropriate to speak of placental ACTH as a substance which causes the adrenal to produce a factor or factors which inhibit hyaluronidase, but it was not necessary to assume that this placental ACTH has the same physiological characteristics as does pituitary ACTH. Furthermore, it was obvious that the observations above recorded, posed a multitude of questions to be answered by further studies before the true relationship between placental and pituitary ACTH can be understood.

VII

The technique of measuring hyaluronidase inhibition has been developed to the point where it can be applied on a semi-quantitative basis as an index of adrenal activity, and as stated, it has been used as the principal type of evidence for the measurement of ACTH and adrenal steroid activity in these series of studies. When inhibition of hyaluronidase-enhanced spreading in the hypophysectomized mouse (or rat) is used as a criterion of ACTH activity, the placental preparations just referred to, possess roughly the same order of activity as Armour's pituitary ACTH, LA-I-A, or Wilson's ACTH (glacial acetic acid extraction); however, using the mobilization of adrenal ascorbic acid, or eosinopenic response as an index of ACTH activity, there would appear to result a wide variation in degree of activity depending on dosages, experimental animals employed, and routes and time intervals of administration of extracts. On the basis of these assays, it would appear that extremely large amounts must be administered in order to obtain a significant decrease in adrenal ascorbic acid; and while the gross impurities of these crude extracts must be considered, nevertheless we have at the same time noted that large doses of chorionic gonadotrophins also have to be used in order to obtain adrenal ascorbic acid decreases that are significant.

(Note: ACTH activity from placental tissue has recently been described in a series of papers by Tarrantino, Pentini, and other Italian workers, and earlier in an isolated study by Jailer and Knowlton; in these studies, depletion of adrenal ascorbic acid has been used as the criterion of ACTH activity. However, the Italian workers have also used liver glycogen deposition, and cholesterol depletion as well as other assay procedures as their basis for estimation of ACTH activity of placental tissues.) Our observations wherein extremely large doses of placental extract preparations are necessary to produce a consistently significant fall in adrenal ascorbic acid, have been noted with placental preparations extracted by different methods (acetone-HCl; acetic acid extraction of acidified acetone dried powders; acetic acid extraction of fresh placental tissue with, and without further purification with oxy-cellulose). At the same time, the hyaluronidase-inhibiting activity of these various preparations have remained constant or have increased, and no destruction of enzyme inhibitory activity has been noted.

Preliminary studies have been carried out relative to adrenal weights in normal and hypophysectomized rats and mice, following placental extract administration; however further investigations are necessary before interpretation of results can be made, as is the case with perfusion studies. However, it is to be noted that repeated administration of placental extracts to both mice and rats (daily over a two week time interval) has produced no adrenal atrophy, no symptoms of toxicity to the animals. It is interesting that small doses of the various placental extract fractions will produce marked hyaluronidase inhibition in hypophysectomized rats, while at the same time, larger doses will show little effect on adrenal ascorbit acid mobilization in similar animals.

Further investigations concerning these as well as other pertinent factors, are to be continued in the near future.

(Further studies not yet completed)

VIII

Preliminary studies carried out in fractionating placental tissue and blood separately, using the modification of the Lyon's method, have suggested that the blood fractions contain a relatively minor quantity of ACTH. Further, that when placentas are washed essentially free of blood immediately after delivery, such placentas can then be stored for appreciable periods of time without loss of hyaluronidase-inhibiting activity in subsequently extracted powders. It would appear that enzymes, or other substances, present in placental blood are responsible for the marked loss of activity in powders extracted from stored combined blood and placental tissues.

Partial purification of the active principle of placental fractions has been attempted using: (1) Astwood's glacial acetic acid method on acetone-dried placental powders, (2) Oxycellulose in the purification of acidified acetone powders, (3) Initial extraction of fresh placental tissue and blood with oxycellulose and, (4) Initial extraction of fresh placental tissue and blood with 1N acetic acid followed by treatment with oxycellulose for varying periods of time. Using Astwood's glacial acetic acid method on acetone-dried placental powders, the final yields were similar to those reported by Astwood on pituitary tissue (10-20%) however, hyaluronidase-inhibition studies on the discarded fractions showed an equal degree of inhibitory activity as did the more purified powders. The resultant powders were essentially comparable in degree of activity to those produced by the modification of the Lyon's method. Studies where oxycellulose was employed in final steps in extractive procedures, yielded powders exhibiting increased activity over those obtained with glacial acetic acid extraction; further, 24-hour dialysis in distilled water of liquid fractions eluted from oxycellulose (using 0.1N HCl) caused no resulting loss of activity. The initial extraction of fresh placental tissue and blood with oxycellulose, or with 1N acetic acid

followed by treatment with oxycellulose, offered no particular advantages in terms of yield or final activity of placental powders.

Results of preliminary purification procedures may be summarized by stating that hyaluronidase-inhibiting activity of placental ACTH is not destroyed by treatment as outlined above; that hyaluronidase-inhibitory activity is enhanced by treatment with oxycellulose but not by treatment with acetic acid alone; that activity is not lost by the dialysis of oxycellulose eluates, but that no marked degree of purification, beyond that obtained with the modified Lyon's procedure, appeared to have taken place. Testing of these fractions was carried out using hypophysectomized, normal, and adrenalectomized mice and rats.

For example: 1 mg. amounts of Armour's ACTH preparation, LA-1-A, routinely produced a 35-50% inhibition of hyaluronidase; 1 mg. amounts of pituitary ACTH extracted by the glacial acetic acid method, produced an average 20-30% inhibition of added hyaluronidase; while Astwood's purified oxycellulose preparation in 1 mg. doses resulted in no definite inhibitory response. These substances were administered intraperitoneally at three hour time intervals prior to hyaluronidase testing. In contrast, following oxycellulose treatment of placental powders, 0.05 mg. amounts produced an average 50% inhibition; 1.0 mg. doses resulted in an average 80% inhibition, whereas 2 mg. doses caused essentially 100% inhibition of added hyaluronidase. (Note: 5 mg. amounts of "Fraction II", extracted by only acidified-acetone procedures, produced the largest yields, and an average consistent 100% inhibition of added hyaluronidase.)

Studies employing Astwood's oxycellulose-purified pituitary ACTH preparation appeared to show quite marked loss of hyaluronidase-inhibiting activity with increasing purification of pituitary ACTH; similarly, this point has been noted to a lesser degree with pituitary preparations extracted by the glacial acetic acid method. These observations are in direct contrast to studies on adrenal ascorbic acid mobilization where ACTH activity of pituitary powders is enhanced following

purification procedures. It is not possible at this time to evaluate the discrepancies noted between mobilization of adrenal ascorbic acid and hyaluronidase-inhibitory activity, and further investigations are necessary; too, the possibility of "permissive" action must be ruled out.

IX

Preliminary investigations for the presence of growth hormone in placental tissues has been made, using the Wilhelmi and Fishman method, and the Astwood glacial acetic acid and oxycellulose method. It would appear on the basis of growth, body weight, and tibial assays in short term experiments, that growth hormone is not produced in appreciable quantities in the human placenta.

Results obtained following the administration of purified growth hormone (supplied us by Drs. Raben and Astwood), clearly showed that growth hormone in 1-10 mg. doses was without hyaluronidase-inhibitory effect.

X

General considerations on the ACTH-adrenal-hyaluronidase relationships.

Over the past several years, we have studied a variety of preparations for their influence in inhibiting the hyaluronidase-enhanced spread of India ink, and their influence on hyaluronic acid metabolism. It has been a consistent observation that such preparations as either ACTH from the pituitary, or crude placental powders, bring about a more marked inhibition of hyaluronidase than is produced by comparable doses of the effective adrenal steroids Compounds E and F when administered systemically. This indicates that stimulation of the adrenals results in a physiological mixture of hormones that is more effective than would be predicted on the basis of individual components, or in the production of inhibitory hormones not thus far studied, i.e., physiological stimulation of the adrenal providing more optimal conditions for such phenomena as hyaluronidase inhibition than can be obtained by the administration of the known effective C-11 steroids. In an attempt

to clarify these factors, further in vivo studies have been carried out, altering the dosages, times of absorption, combination of compounds tested, and other variables in experimental conditions. Compound F, while more slowly absorbed was found to be equally as effective as Compound E when tested following longer time intervals of absorption, and maximal inhibitory effects are maintained over longer time intervals. Studies on the "amorphous" fraction in terms of hyaluronidase-inhibition, indicate no hyaluronidase inhibitory activity to be present, rather when administered in large dosages, it appears to have effects similar to those of DOCA, and causing hyaluronidase-enhanced spreading. Purified growth hormone does not inhibit the hyaluronidase spreading phenomenon.

Investigations from this laboratory have indicated that the activity of the C-11 oxygenated adrenal steroids is concerned, at least in part, with a direct inhibition of the enzyme hyaluronidase thus producing alterations in the permeability of the ground substances. Further experimental establishment of the actual role of the C-11 oxygenated steroids in the spreading reaction is of considerable importance, in that it underlies the action of physiological stimulation by primary or stress substances, as well as by pituitary or placental ACTH.

The fact that pituitary ACTH is far more active than placental extracted powders as measured by adrenal ascorbic acid mobilization, while on the other hand, these same two preparations are of approximately equal activity in inhibition of the spreading reaction, clearly points out the necessity of further studies as to the mode of action of placental ACTH. Measurements of their comparative activity on adrenal weights and histochemical alterations in the adrenal cortex may provide further clues as to their difference in effects on the adrenal cortex, together with studies on liver glycogen deposition and adrenal cholesterol depletion. As stated, the question of permissive action must be investigated.

The possibility that placental ACTH would have a practical therapeutic value may be considered inasmuch as: 1. Administration in large doses, and over

repeated time intervals, causes no evident symptoms of toxicity; 2. In that it is of human origin, it presumably would not cause a foreign protein reaction; 3. Similar to pituitary ACTH, it produces a marked inhibition of hyaluronidase in the absence of the pituitary but presence of the adrenal; 4. It produces no adrenal atrophy; 5. As may be seen from the work of Hench in studies on pregnancy, the work of Tarrantino, Pentini, Granier, and others, it would appear implicated in the noted improvement in arthritic and other connective tissue disease processes; 6. It is available in large quantities.

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